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Nitric oxide-induced calcium release via ryanodine receptors regulates neuronal function

Sho Kakizawa, Toshiko Yamazawa, Yili Chen, Akihiro Ito,Takashi Murayama, Hideto Oyamada, Nagomi Kurebayashi, Osamu Sato, Masahiko Watanabe, Nozomu Mori, Katsuji Oguchi, Takashi Sakurai, Hiroshi Takeshima, Nobuhito Saito and Masamitsu Iino

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1st Editorial Decision 05 July 2011

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see, the referees find the analysis interesting and suitable for publication here. Referee #1 has no further comments, while referee #2 and 3 raise a few issues that should be resolved. Given these comments, I would like to invite you to submit a revised version of the manuscript that addresses the concerns raised. I should add that it is EMBO Journal policy to allow only a single round of revision and that it is therefore important to address the concerns raised at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

It is very clear that a huge amount of work went into this manuscript which describes the functional consequences of S-nitrosylation of RyR1 on Ca2+ signaling and neuronal function. The authors convincingly demonstrate that S-nitrosylation induces Ca2+ release via RyR1 and that the nitrosylation is driven by activity dependent NOS 1 activation. Furthermore they show that this NO driven Ca2+ release (which they designate NICR) is involved in neuronal cell death.

This is a well written manuscript describing a massive effort to demonstrate RyR1 S-nitrosylation and its functional consequences in the brain. The experiments appear to be carefully preformed and this paper is likely to have a major impact on the field.

Referee #2

These interesting and informative studies report on the regulation of neuronal RyR1 by NO-mediated nitrosylation. Although regulation of single RyR1 and RyR1 function in skeletal muscle by nitrosylation is well established, this study reveals similar regulation in neurons and demonstrates its physiological relevance. The key finding is the activation of Nitrosylation-induced Ca2+ release (NICR) by physiological patterns of neuronal activity that explains the role of NO and RyR1-mediated Ca2+ release in LTP. However, the weakest point of the studies is in establishing the specific role of nitrosylation in the in vivo effects that can be addressed as suggested below. In addition, several controls are missing.

1. Although it is shown that brain RyR1 is nitrosylated in response to neuronal stimulation, it is not certain that this plays a role in Ca2+ release activated by BS. To show this nitrosylation needs to be manipulated and showing that such manipulation affect nitrosylation, Ca2+ release and LTP. One way to do so is to inhibit xanthine oxidase with oxypurine or any other inhibitor of the oxidase. Any other means of manipulation nitrosylation in vivo that the authors can come with should further strengthen the manuscript and conclusion.

2. To ensure that deletion of NOS1 has no effect beyond inhibition of NO production, a required control is to show that NO donors can activate RyR1-mediated Ca2+ release in NOS1-/- cells in a manner similar to that seen in WT cells.

3. Similarly, do NO donors recover LTP induced by BS in NOS1-/- neurons?

4. It is not clear why the authors used the less specific dantrolene rather than the more common ryanodine to inhibit RyR-mediated functions, in particular when using isolated cells. At high concentrations dantrolene can also inhibit CICR and SOC-mediated Ca2+ influx.

5. In page 15, lines 2-4 the authors state "The agonistic effect of NO on neuronal intracellular Ca2+ release is mediated by S-nitrosylation of RyR1, in particular at cysteine 3635 of the Ca2+ release channel". The authors never demonstrated a role of cysteine 3635 in vivo. There is no evidence to show that cysteine 3635 is the specific cysteine nitrosylated in the brain/neurons during stimulation. The sentence should be revised.

Referee #3

Nitric oxide-induced calcium release via ryanodine receptors regulates neuronal function
Kakizawa, Toshiko Yamazawa, Yili Chen, Akihiro Ito, Nobuhito Saito, Takashi Murayama, Nagomi Kurebayashi, Osamu Sato, Takashi Sakurai, Hideto Oyamada, Katsuji Oguchi, Masahiko Watanabe, Nozomu Mori, Hiroshi Takeshima and Masamitsu Iino

Kakizawa et al. found that nitric oxide (NO) induces Ca2+ release from type 1 ryanodine receptors (RyR1) of endoplasmic reticulum in Purkinje cells of cerebellar slice preparations. By using mutated RyR1, which was exogenously expressed in HEK293 cells, they demonstrated that the Ca2+ release

is mediated via S-nitrosylation of RyR1 at cysteine 3635. Further, authors tested the involvement of this pathway of Ca2+ release in NO-dependent phenomena in neurons, cerebellar LTP, ischemic brain injury, and neuronal cell death.
Although the enhancement of open probability of RyR1 by S-nitrosylation was reported, Kakizawa et al. showed for the first time that this mechanism works in live cells upon NO donor treatment. In addition, they demonstrated physiological or pathological relevance of NO-triggered Ca2+ release from RyR1 via S-nitrosylation in neurons. Thus, their results provide a new interesting pathway connecting NO signal and Ca2+ signal, both of which are important for several neuronal functions. However, I believe that authors have to clarify a few points, in order to conclude that this pathway is involved in cerebellar LTP and neuronal cell death.

<Major points>
(1) As authors referred, it was reported that cerebellar LTP is inhibited by 30 mM BAPTA, so that a certain level of Ca2+ concentration seems to be required for LTP. However, LTP can be induced by 1Hz train of single parallel fiber stimulation and the increase in Ca2+ concentration was not so far observed by such stimulation. Indeed, a report showed that LTP is observed in the presence of 5 mM BAPTA but is reduced in the absence of BAPTA (Lev-Ram V. et al., Proc Natl Acad Sci USA, 2002 99:8389). Another report showed that LTP is induced in the presence of 20 mM BAPTA by a type of stimulation, which normally induces LTD (Coesmans M. et al. Neuron, 2004 44:691). These results are not in line with the idea that slow and clear increase in Ca2+ concentration is required for LTP. Thus, I wonder how authors reconcile these results with their idea.

(2) Related to the comment (1), I would like to know the following results.
a. Does lower concentration of BAPTA (5-20 mM) inhibit neither BS-induced Ca2+ increase nor LTP?
b. Authors showed that thapsigargin, 30 mM BAPTA, and dantrolene blocked LTP. Do they affect basal level of Ca2+ concentration?

(3) In the last paragraph of discussion, authors describe the difficulty of measuring S-nitrosylation of RyR1 in cerebral cortex. I think that authors could test the involvement of S-nitrosylation at least in in vitro system, such as analysis of cell death using cerebral culture neurons, shown in Figure 6. In the earlier experiments (Figure 2), authors demonstrated clear difference in NO-induced Ca2+ release between HEK293 cells expressing exogenous RyR1 and C3635S-RyR1. Thus, I wonder if expression of RyR1, but not C3635S-RyR1, in RyR1-/- neurons restores NO-dependent Ca2+ increase and cell death.

<Minor points>
(4) Authors examined S-nitrosylation by biotin-switch method. Is the signal of S-nitrosylation specific to RyR1? If so, I assume that authors performed immunoprecipitation by using a RyR1 antibody. However, it is not mentioned in the "materials and methods" or "Supplementary materials".

(5) At the first paragraph of discussion, authors mentioned about the slow and prolonged time course of NO-dependent Ca2+ release from RyR1 in neurons. The time course of Ca2+ release looks slightly different between HEK293 cells expressing RyR1 (Figure 2C) and neuronal cells (Figure 1B, 1C, 1F, Figure 6A, 6B). Thus, the slow and prolonged Ca2+ release in neurons may not only due to the signaling pathway, but also due to some aspects in neurons. The time course of Ca2+ release in HEK293 cells seems to be consistent with time course of S-nitrosylation (Figure 2F). I feel that it would be nice if authors add a discussion why NO-dependent Ca2+ release from RyR1 is slow and prolonged specifically in neurons.

1st Revision - authors' response 09 September 2011

Referee #2

These interesting and informative studies report on the regulation of neuronal RyR1 by NO-mediated nitrosylation. Although regulation of single RyR1 and RyR1 function in skeletal muscle by

nitrosylation is well established, this study reveals similar regulation in neurons and demonstrates its physiological relevance. The key finding is the activation of Nitrosylation-induced Ca^{2+} release (NICR) by physiological patterns of neuronal activity that explains the role of NO and RyR1-mediated Ca^{2+} release in LTP. However, the weakest point of the studies is in establishing the specific role of nitrosylation in the in vivo effects that can be addressed as suggested below. In addition, several controls are missing.

1. Although it is shown that brain RyR1 is nitrosylated in response to neuronal stimulation, it is not certain that this plays a role in Ca^{2+} release activated by BS. To show this nitrosylation needs to be manipulated and showing that such manipulation affect nitrosylation, Ca^{2+} release and LTP. One way to do so is to inhibit xanthine oxidase with oxypurine or any other inhibitor of the oxidase. Any other means of manipulation nitrosylation in vivo that the authors can come with should further strengthen the manuscript and conclusion.

The reviewer requested additional experiments to show the involvement of *S*-nitrosylation in BS-induced Ca^{2+} release and LTP using a method to manipulate *S*-nitrosylation. Ascorbic acid has been used for selective reduction of cysteine *S*-nitrosothiols (Burgoyne & Eaton, *Meth. Enzymol.* 473: 281–303, 2010; Jaffrey et al. *Nat. Cell Biol.* 3: 193–197, 2001), so we included 10 mM ascorbic acid in the patch pipette and examined its effect. Ascorbic acid inhibited BS-induced Ca^{2+} release and LTP (Figure S3J and S4H). This is mentioned in the revised manuscript (page 10, bottom line – page 11, line 3; page 12, lines 17–18).

2. To ensure that deletion of NOS1 has no effect beyond inhibition of NO production, a required control is to show that NO donors can activate RyR1-mediated Ca^{2+} release in NOS1-/- cells in a manner similar to that seen in WT cells.

Figure S3M in the original manuscript showed that NOC7 activates RyR1-mediated Ca^{2+} release in $Nos1^{-/-}$ cells. We have extended this measurement, and now compare NICR in $Nos1^{+/+}$ and $Nos1^{-/-}$ Purkinje cells at 30 and 300 μM NOC7 concentrations in the revised manuscript (Figure S3P). The results indicate that NO-induced Ca^{2+} release is intact in $Nos1^{-/-}$ Purkinje cells. This is mentioned in the revised manuscript (page 10, lines 17–19).

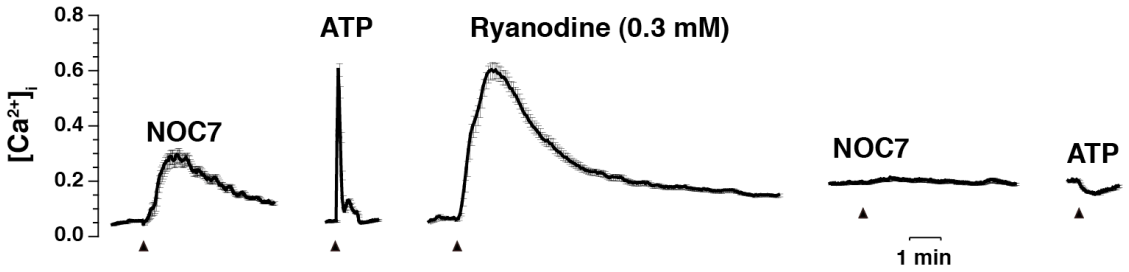
3. Similarly, do NO donors recover LTP induced by BS in NOS1-/- neurons?

We carried out new experiments that showed that 30 μM NOC7 induces LTP in $Nos1^{-/-}$ cerebellar slice preparations as in $Nos1^{+/+}$ slices (Figure S4L). Thus, NO-induced LTP generation is also intact in $Nos1^{-/-}$ Purkinje cells. This is mentioned in the revised manuscript (page 12, lines 3–4).

4. It is not clear why the authors used the less specific dantrolene rather than the more common ryanodine to inhibit RyR-mediated functions, in particular when using isolated cells. At high concentrations dantrolene can also inhibit CICR and SOC-mediated Ca^{2+} influx.

Ryanodine has a complex effect on RyR function depending on its concentration (for review, see Sutko, *Pharmacol. Rev.* 49, 53–98, 1997). At lower concentrations (nM–μM), ryanodine binding to RyR fixes the Ca^{2+} release channel in a continuously open subconductance state ("open-lock"). Open-locked RyRs provide a Ca^{2+} leakage pathway from the Ca^{2+} store, and therefore Ca^{2+} stores become depleted. Ryanodine at very high concentrations (sub-mM and over) closes RyR. Therefore, Ca^{2+} release from the store via RyR can be blocked by ryanodine at low or high concentrations, but by different mechanisms. The different modes of action can make interpretations of the results complex. When RyRs are open-locked, Ca^{2+} release via IP_3R may be also blocked, because the Ca^{2+} store is depleted. We examined if we can close RyR without depleting the store using 0.3 mM ryanodine in HEK293 cells (See Box 1 below). We found that ryanodine treatment itself induced a large transient increase in $[Ca^{2+}]_i$. After the transient increase in $[Ca^{2+}]_i$, the resting Ca^{2+} concentrations remained elevated. Under this condition, NOC7-induced Ca^{2+} release was blocked. However, it also blocked ATP (a purinergic receptor agonist)-induced Ca^{2+} release via the IP_3R, indicating that the Ca^{2+} store is depleted. Thus, the effect of ryanodine is similar to the store depletion caused by thapsigargin. Under this experimental condition, we cannot discriminate whether RyR or IP_3R is involved in NICR. Much higher ryanodine concentrations (for example, 1–10 mM) may allow us to block RyR without depleting the Ca^{2+} store, but it is not practical, especially for experiments using brain slice preparations in which drug concentrations increase

gradually due to the thickness (diffusion path) of slices. Furthermore, such high concentrations of ryanodine may raise a new problem regarding drug specificity. Thus, in practice, selectively closing RyR using ryanodine in our experiments is difficult. We therefore used dantrolene, which does not block IP_3R.



Box 1. Effect of ryanodine treatment on the Ca^{2+} response to NOC7 (1 mM) and ATP (10 μM) in HEK293 cells expressing exogenous RyR1. ATP responses of similar magnitude are observed in the absence of extrinsic RyR1 expression (data not shown). NICR and purinergic responses are blocked after ryanodine (0.3 mM) treatment. Mean ± s.e.m. n = 50–60 cells.

5. In page 15, lines 2-4 the authors state "The agonistic effect of NO on neuronal intracellular Ca^{2+} release is mediated by S-nitrosylation of RyR1, in particular at cysteine 3635 of the Ca^{2+} release channel". The authors never demonstrated a role of cysteine 3635 in vivo. There is no evidence to show that cysteine 3635 is the specific cysteine nitrosylated in the brain/neurons during stimulation. The sentence should be revised.

The first paragraph in the Discussion has been revised to conform to the comment of the referee (page 16, lines 4–6).

Referee #3

Kakizawa et al. found that nitric oxide (NO) induces Ca^{2+} release from type 1 ryanodine receptors (RyR1) of endoplasmic reticulum in Purkinje cells of cerebellar slice preparations. By using mutated RyR1, which was exogenously expressed in HEK293 cells, they demonstrated that the Ca^{2+} release is mediated via S-nitrosylation of RyR1 at cysteine 3635. Further, authors tested the involvement of this pathway of Ca^{2+} release in NO-dependent phenomena in neurons, cerebellar LTP, ischemic brain injury, and neuronal cell death.
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stimulation, which normally induces LTD (Coesmans M. et al. Neuron, 2004 44:691). These results are not in line with the idea that slow and clear increase in Ca^{2+} concentration is required for LTP. Thus, I wonder how authors reconcile these results with their idea.

The referee questions the relationship between NICR and LTP. To address this comment, we first measured the intracellular Ca^{2+} concentration in the dendrites of Purkinje cells receiving a 1-Hz train of single parallel fiber stimulation for 5 min, because this measurement has not been carried out. We first confirmed that the single-pulse protocol induces an induction of LTP (Figure S4K). We then carried out Ca^{2+} imaging, and found a clear increase in the intracellular Ca^{2+} concentration in response to a 1-Hz train of single parallel fiber stimulation (Figure S3L). Although the time course of Ca^{2+} increase was slower than that induced by BS, the peak amplitude was comparable with that induced by BS. Therefore, single-pulse stimulation and BS generate an increase in intracellular Ca^{2+}. This has been added to the revised manuscript on page 11, lines 16–18.

We next considered the effect of BAPTA on LTP. Lev-Ram et al. showed that LTP is observed in the presence of 5 mM BAPTA but is reduced in the absence of BAPTA (*Proc. Natl. Acad. Sci. USA*, 99: 8389–8393, 2002). The apparent potentiating effect of 5 mM BAPTA on LTP could be explained by the prevention of residual long-term depression (LTD), as suggested by those authors. Although LTP is induced in the presence of 20 mM BAPTA by a type of stimulation, which normally induces LTD (Coesmans et al. *Neuron*, 44: 691–700, 2004), it has not been shown whether or not 20 mM BAPTA inhibits LTP induced by authentic LTP protocols. In our original submission, we used 30 mM BAPTA for Ca^{2+} buffering inside PCs to block LTP because it was the concentration used by the Coesmans et al. As suggested by the referee (see our response to the next comment), we carried out new LTP experiments using 5, 10, 20, and 30 mM BAPTA in the pipette. We found a dose-dependent inhibition of LTP and that 10 mM BAPTA was almost sufficient to inhibit LTP (Figure S4J). The results are in general agreement with the notion of involvement of Ca^{2+} in the generation of LTP, but the absence of an effect of 5 mM BAPTA on LTP deserves explanation. Although the rate of rise of Ca^{2+} during BS-induced NICR in PCs is low (i.e. the onset of RyR opening events is asynchronous within the cell), individual Ca^{2+} release events via RyR1 provide a massive Ca^{2+} release in close vicinity of the Ca^{2+}-release channel. Indeed, the single-channel current via RyR is estimated to be ~0.5 pA under near-physiological conditions (Kettlun et al. *J. Gen. Physiol.* 122, 407–417, 2003). If the target molecules of Ca^{2+} release localize close to the Ca^{2+}-release channel, it seems possible that Ca^{2+} binding to the target molecules cannot be completely blocked by lower concentrations of BAPTA. A recent study (Faas et al. *Nat. Neurosci.* 14, 301–304, 2011) showed that the Ca^{2+} binding rate constants of calmodulin are so high that calmodulin can effectively "trap" Ca^{2+} immediately when Ca^{2+} enters the cytoplasm before binding to cytoplasmic Ca^{2+} buffers. Therefore, if calmodulin or other Ca^{2+}-binding proteins with similar Ca^{2+}-binding properties are involved in LTP, it seems possible that lower concentrations of BAPTA are unable to block the process leading to LTP. For example, Ca^{2+} release via RyR can be a signal to other adjacent molecules in the presence of BAPTA in cardiac cells. L-type Ca^{2+} channels undergo inactivation via a Ca^{2+}-calmodulin-dependent mechanism. In cardiac cells, RyR2 and L-type Ca^{2+} channels localize at the junction between the sarcoplasmic reticulum and plasma membrane, and Ca^{2+} release from RyR2 induces Ca^{2+}-induced inactivation of L-type Ca^{2+} channels on the plasma membrane. Sham (*J. Physiol.* 500, 285–295, 1997) studied the effect of 3 and 10 mM BAPTA on Ca^{2+} release-induced inactivation and found that a considerable fraction of Ca^{2+} release-dependent inactivation remains even in the presence of BAPTA: about 61% and 32% of control with 3 and 10 mM BAPTA, respectively, at –20 mV. This reduction includes the inhibition of RyR activation by Ca^{2+} influx via L-type channels, and the real effect of BAPTA on Ca^{2+} release-induced inactivation of L-type Ca^{2+} channels should be smaller. Taken together, the present results suggest that NICR regulates LTP generation via local Ca^{2+} signaling. Of course the molecular mechanism of the local signaling requires further investigation. These new results and our discussion of them have been added to the revised manuscript (page 12, bottom line – page 13, line 2; page 18, line 8 – page 19, line 16).

(2) Related to the comment (1), I would like to know the following results.
a. Does lower concentration of BAPTA (5-20 mM) inhibit neither BS-induced Ca^{2+} increase nor LTP?

We carried out new LTP experiments at 5, 10, 20, and 30 mM BAPTA (Figure S4J). In accordance with the previous results, 5 mM-BAPTA did not inhibit LTP, but higher BAPTA concentrations inhibited LTP (see our response above). The results are explained in the revised manuscript (pages

12–13). The concentration of the Ca^{2+} indicator OGB1 that we used was 0.1 mM. OGB1 is a BAPTA derivative, so BAPTA competes with OGB1 for Ca^{2+} binding (i.e. BAPTA is a competitive inhibitor of OGB1). Therefore, in the presence of, for example, 10 mM BAPTA, the magnitude of the OGB1 signal becomes ~1/100 of that without BAPTA. This effect precludes the Ca^{2+} measurement using OGB1 in the presence of BAPTA.

b. Authors showed that thapsigargin, 30 mM BAPTA, and dantrolene blocked LTP. Do they affect basal level of Ca^{2+} concentration?

OGB1, a non-ratiometric Ca^{2+} indicator used in slice preparations, does not allow precise estimation of absolute values of the resting Ca^{2+} concentration. In response to the referee's comment, we carried out ratiometric Ca^{2+} measurements using fura-2 in cultured Purkinje cells. We found no significant effect of dantrolene on the resting Ca^{2+} concentration: before dantrolene, 52.1 ± 12.8 nM; after dantrolene, 55.6 ± 14.0 nM (mean ± s.d., n = 22, P = 0.398). Thapsigargin increased the resting Ca^{2+} concentration from 56.3 ± 10.3 nM to 70.0 ± 16.4 nM (n = 12, P = 0.0287). This was probably due to store-operated Ca^{2+} entry. We also observed the effect of BAPTA by loading BAPTA AM for 30 min at 20 μM concentration, which is tenfold greater than that of fura-2 AM. It reduced the resting Ca^{2+} concentration from 53.5 ± 11.0 nM to 41.8 ± 12.5 nM (n = 31, P = 0.0003). BAPTA introduces additional Ca^{2+} buffer to the cytoplasm, but its effect on the resting Ca^{2+} concentration will be partly compensated by Ca^{2+} influx. Thus, even though thapsigargin, BAPTA and dantrolene block LTP, their effects on the resting Ca^{2+} concentration were not constant. We therefore feel that the LTP-blocking effect of these manipulations is unlikely to be caused by changes in the resting Ca^{2+} concentration.

(3) In the last paragraph of discussion, authors describe the difficulty of measuring S-nitrosylation of RyR1 in cerebral cortex. I think that authors could test the involvement of S-nitrosylation at least in in vitro system, such as analysis of cell death using cerebral culture neurons, shown in Figure 6. In the earlier experiments (Figure 2), authors demonstrated clear difference in NO-induced Ca^{2+} release between HEK293 cells expressing exogenous RyR1 and C3635S-RyR1. Thus, I wonder if expression of RyR1, but not C3635S-RyR1, in RyR1-/- neurons restores NO-dependent Ca^{2+} increase and cell death.

To adequately respond to the referee's comment, we searched for a method to introduce the *Ryr1* gene to primary cultures of *Ryr1*$^{-/-}$ neurons. However, due to the large size of the *Ryr1* gene (>15 kbp), efficient transfection was difficult. The Lenti virus or AAV cannot harbor the *Ryr1* gene because of its large size. We thus used the HSV-1 virus, which can carry the *Ryr1* gene (Wang et al. *Am. J. Physiol.* 278**:** C619–C626, 2000), but a sufficient rate of infection has not been obtained so far. To circumvent these problems in primary cultured neurons, we examined cell viability in HEK cells expressing RyR1 or C3635A-RyR1. NOC12 induced a loss in cell viability in HEK cells expressing wild-type RyR1. This decreased cell viability was partly reversed by dantrolene. NOC12-induced cell-viability decrease was milder in HEK cells expressing C3635A RyR1, and in those cells the protective effect of dantrolene was absent. These results (now shown in Figure S5 and explained in the revised manuscript on page 15, 2nd paragraph) support the notion that *S*-nitrosylation-dependent NICR has an adverse effect on cell viability.

<Minor points>
(4) Authors examined S-nitrosylation by biotin-switch method. Is the signal of S-nitrosylation specific to RyR1? If so, I assume that authors performed immunoprecipitation by using a RyR1 antibody. However, it is not mentioned in the "materials and methods" or "Supplementary materials".

Detection of RyR1 was carried out by western blotting. Details of the method (including the antibodies used) are described in Supplementary Materials and Methods (page 4). Furthermore, we added a new result (Figure S2A), in which *S*-nitrosylation was observed only when RyR1 expression was induced. This result provides additional support for the notion of *S*-nitrosylation of RyR1.

(5) At the first paragraph of discussion, authors mentioned about the slow and prolonged time course of NO-dependent Ca^{2+} release from RyR1 in neurons. The time course of Ca^{2+} release looks slightly different between HEK293 cells expressing RyR1 (Figure 2C) and neuronal cells (Figure 1B, 1C, 1F, Figure 6A, 6B). Thus, the slow and prolonged Ca^{2+} release in neurons may not only due

to the signaling pathway, but also due to some aspects in neurons. The time course of Ca^{2+} release in HEK293 cells seems to be consistent with time course of S-nitrosylation (Figure 2F). I feel that it would be nice if authors add a discussion why NO-dependent Ca^{2+} release from RyR1 is slow and prolonged specifically in neurons.

We wished to compare the time-course of neuronal NICR in response to synaptic input (Figure 3G) with that of Ca^{2+} transient of a twitch or a tetanus in muscle cells (which has much greater rates of rise and fall). For example, the Ca^{2+} transient during a twitch contraction is completed within a fraction of a second. This point is now made clear in the revised manuscript (page 16, lines 13–16).

We would also like to provide further explanation about the apparent difference in the time course of NO donor-induced Ca^{2+} release between neurons and HEK cells. In the HEK cell experiments (Figure 2), NOC7 was applied to the cultured cells using a puffer pipette. Therefore, NOC7 had direct access to the cell surface. In the Purkinje cell experiments (Figure 1), NOC7 was superfused over the slice preparation and we imaged Purkinje cells 10–50 μm-deep from the surface of slice preparations. The arrival of NOC7 to Purkinje cells in this case is delayed by the time required for the exchange of solution within the measuring chamber and by the diffusion of NOC7 through the cerebellar tissue. Therefore, the apparent difference in the time-course of NICR between Purkinje cells and HEK cells may be accounted for by the difference in the time-course of increase in NOC7 concentration. In the experiments using cerebral neurons in culture (Figure 6), NOC12 instead of NOC7 was used as a NO donor. The half-lives of NOC7 and NOC12 are 20–30-times different, so the time-course of change in NO concentration is prolonged if NOC12 is used as an NO donor. This precludes direct comparison of the results between neurons and HEK cells. We added a new result showing the time-course of NICR when 300 μM NOC7 was applied to cultured cerebral neurons (Figure S1H). The NICR time-course was similar to that of HEK cells challenged with the same concentration of NOC7 (Figure S2G, top left panel). Therefore, we did not find a major difference in the NICR time-course between neurons and HEK cells under comparable conditions. However, we do not rule out the possibility that NICR time courses are dependent upon the cell type. The time courses are likely to be influenced by many factors. These include the time course of increase in NO concentration, expression levels of RyR1 and SERCA, and the rate of removal of *S*-nitrosylation. This statement has been added to the first paragraph of the Discussion (page 16, lines 17–19).

2nd Editorial Decision 26 September 2011

Thank you for submitting your revised manuscript to the EMBO Journal. Your study has now been seen by the original referee #3 and his/her comments are provided below. As you can see, the referee appreciates the introduced changes and supports publication in the EMBO Journal. There is one minor point that has to be attended to before acceptance here - see below. The remaining point can be addressed with appropriate text changes no further experiments are needed. Once we receive the revised version we will proceed with the acceptance of the paper for publication here.

Thank you for submitting your interesting study to the EMBO Journal

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORT

Referee #3

Authors performed several additional experiments and described these experiments or discussions in the revised manuscripts. Most of them are carefully and nicely addressed. Therefore I am almost satisfied with this version of manuscript. However, I believe that one point, which is related to my previous comments (1) and (2)-a, has to be addressed.
The dose-dependent inhibition of LTP by BAPTA was nicely demonstrated. However, results

obtained with 5 mM BAPTA are not precisely described. Authors acknowledged in their response that the Ca measurement in the presence of BAPTA with OGB1 in Purkinje cells is not possible, because of the low signal of OGB1, indicating low concentrations of Ca. This means that the Ca increase by BS in the presence of 5 mM BAPTA was, if any, undetectable, while LTP was intact. Even though results using dantrolene, thapsigargin, and higher concentrations of BAPTA indicate that NICR from RyR is involved in LTP, the results of 5 mM BAPTA reveal that clear Ca increase shown in Figure 3 is not required for LTP. This point is important, but the current version of manuscript is misleading. In addition to the discussion about localized Ca release from RyR (page 18-19), the results of 5 mM BAPTA has to be described and the conclusion has to be modified accordingly in the result section (page 13).

2nd Revision - authors' response 27 September 2011

Referee #3

Authors performed several additional experiments and described these experiments or discussions in the revised manuscripts. Most of them are carefully and nicely addressed. Therefore I am almost satisfied with this version of manuscript. However, I believe that one point, which is related to my previous comments (1) and (2)-a, has to be addressed.
The dose-dependent inhibition of LTP by BAPTA was nicely demonstrated. However, results obtained with 5 mM BAPTA are not precisely described. Authors acknowledged in their response that the Ca measurement in the presence of BAPTA with OGB1 in Purkinje cells is not possible, because of the low signal of OGB1, indicating low concentrations of Ca. This means that the Ca increase by BS in the presence of 5 mM BAPTA was, if any, undetectable, while LTP was intact. Even though results using dantrolene, thapsigargin, and higher concentrations of BAPTA indicate that NICR from RyR is involved in LTP, the results of 5 mM BAPTA reveal that clear Ca increase shown in Figure 3 is not required for LTP. This point is important, but the current version of manuscript is misleading. In addition to the discussion about localized Ca release from RyR (page 18-19), the results of 5 mM BAPTA has to be described and the conclusion has to be modified accordingly in the result section (page 13).

We described the absence of effect of 5 mM BAPTA on LTP in the revised Results, and modified the conclusion, which now states that Ca^{2+} release via the RyR1 (rather than a global increase in $[Ca^{2+}]_i$) is required for the induction of LTP. See Page 13, Lines 2–7. We also added a statement that 5 mM BAPTA is expected to inhibit a global increase in the dendritic $[Ca^{2+}]_i$ in Discussion. See Page 18, Lines 13–14.

